

MAMMALIAN T-TYPE CALCIUM CHANNELS

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25 February 1998 which claims priority from Provisional Application No. 60/039,204
5 filed 28 February 1997. The disclosures of these applications are incorporated by
reference herein.

TECHNICAL FIELD

The invention relates to T-type calcium channel encoding sequences, expression
of these sequences, and methods to screen for compounds which antagonize calcium
10 channel activity. The invention is also related to molecular tools derived from knowledge
of the molecular structure of T-type calcium channels.

BACKGROUND OF THE INVENTION

The rapid entry of calcium into cells is mediated by a class of proteins called
voltage-gated calcium channels. Calcium channels are a heterogeneous class of
15 molecules that respond to depolarization by opening a calcium-selective pore through the
plasma membrane. The entry of calcium into cells mediates a wide variety of cellular
and physiological responses including excitation-contraction coupling, hormone secretion
and gene expression. In neurons, calcium entry directly affects membrane potential and
contributes to electrical properties such as excitability, repetitive firing patterns and
20 pacemaker activity. Miller, R.J. (1987) "Multiple calcium channels and neuronal
function." *Science* 235:46-52. Calcium entry further affects neuronal functions by
directly regulating calcium-dependent ion channels and modulating the activity of
calcium-dependent enzymes such as protein kinase C and calmodulin-dependent protein
kinase II. An increase in calcium concentration at the presynaptic nerve terminal triggers
25 the release of neurotransmitter. Calcium entry also plays a role in neurite outgrowth and
growth cone migration in developing neurons and has been implicated in long-term
changes in neuronal activity.

In addition to the variety of normal physiological functions mediated by calcium channels, they are also implicated in a number of human disorders. Recently, mutations identified in human and mouse calcium channel genes have been found to account for several disorders including, familial hemiplegic migraine, episodic ataxia type 2, cerebellar ataxia, absence epilepsy and seizures. Fletcher, *et al.* (1996) "Absence epilepsy in tottering mutant mice is associated with calcium channel defects." *Cell* 87:607-617; Burgess, *et al.* (1997) "Mutation of the Ca²⁺ channel β subunit gene Cchb4 is associated with ataxia and seizures in the lethargic (lh) mouse." *Cell* 88:385-392; Ophoff, *et al.* (1996) "Familial hemiplegic migraine and episodic ataxia type-2 are caused by mutations in the Ca²⁺ channel gene CACNL1A4." *Cell* 87:543-552; Zhuchenko, O., *et al.* (1997) "Autosomal dominant cerebellar ataxia (SCA6) associated with the small polyglutamine expansions in the α 1A-voltage- dependent calcium channel." *Nature Genetics* 15:62-69.

The clinical treatment of some disorders has been aided by the development of therapeutic calcium channel antagonists. Janis, *et al.* (1991) in *Calcium Channels: Their Properties, Functions, Regulation and Clinical Relevance*. CRC Press, London.

Native calcium channels have been classified by their electrophysiological and pharmacological properties as T, L, N, P and Q types (for reviews see McCleskey, *et al.* (1991) "Functional properties of voltage-dependent calcium channels." *Curr. Topics Membr.* 39: 295-326, and Dunlap, *et al.* (1995) "Exocytotic Ca²⁺ channels in mammalian central neurons." *Trends Neurosci.* 18:89-98.). T-type (or low voltage-activated) channels describe a broad class of molecules that activate at negative potentials and are highly sensitive to changes in resting potential. The L, N, P and Q-type channels activate at more positive potentials and display diverse kinetics and voltage-dependent properties. There is some overlap in biophysical properties of the high voltage-activated channels, consequently pharmacological profiles are useful to further distinguish them. L-type channels are sensitive to dihydropyridine (DHP) agonists and antagonists, N-type channels are blocked by the *Conus geographus* peptide toxin, ω -conotoxin GVIA, and P-type channels are blocked by the peptide ω -agatoxin IVA from the venom of the funnel web spider, *Agelenopsis aperta*. A fourth type of high voltage-activated Ca channel (Q-type) has been described, although whether the Q- and P-type channels are distinct molecular entities is controversial (Sather *et al.* (1993) "Distinctive biophysical and

pharmacological properties of class A (B1) calcium channel α_1 subunits." *Neuron* 11:291-303; Stea, *et al.* (1994) "Localization and functional properties of a rat brain α_1A calcium channel reflect similarities to neuronal Q- and P-type channels." *Proc Natl Acad Sci (USA)* 91:10576-10580; Bourinet, E., *et al.* (1999) *Nature Neuroscience* 2:407-415).
5 Several types of calcium conductances do not fall neatly into any of the above categories and there is variability of properties even within a category suggesting that additional calcium channels subtypes remain to be classified.

Biochemical analyses show that neuronal high-threshold calcium channels are heterooligomeric complexes consisting of three distinct subunits (α_1 , $\alpha_2\delta$ and β)
10 (reviewed by De Waard, *et al.* (1997) in *Ion Channels*, Volume 4, edited by Narahashi, T. Plenum Press, New York). The α_1 subunit is the major pore-forming subunit and contains the voltage sensor and binding sites for calcium channel antagonists. The mainly extracellular Alternatively, the α_2 subunit is disulphide-linked to the transmembrane δ subunit and both are derived from the same gene and are proteolytically
15 cleaved *in vivo*. The β subunit is a non-glycosylated, hydrophilic protein with a high affinity of binding to a cytoplasmic region of the α_1 subunit. A fourth subunit, γ , is unique to L-type Ca channels expressed in skeletal muscle T-tubules. The isolation and characterization of γ -subunit-encoding cDNAs is described in U.S. Patent No. 5,386,025 which is incorporated herein by reference.

Molecular cloning has revealed the cDNA and corresponding amino acid sequences of six different types of α_1 subunits (α_{1A} , α_{1B} , α_{1C} , α_{1D} , α_{1E} and α_{1S}) and four types of β subunits (β_1 , β_2 , β_3 and β_4) (reviewed in Stea, A., Soong, T.W. and Snutch, T.P. (1994) "Voltage-gated calcium channels." in *Handbook of Receptors and Channels*. Edited by R.A. North, CRC Press). A comparison of the amino acid
20 sequences of these α_1 subunits is included in this publication, which is incorporated herein by reference. PCT Patent Publication WO 95/04144, which is incorporated herein by reference, discloses the sequence and expression of α_{1E} calcium channel subunits.

As described in Stea, A., *et al.* (1994) (*supra*), the α_1 subunits are generally of the order of 2000 amino acids in length, ranging from 1873 amino acids in α_{1S} derived from
30 rabbit to 2424 amino acids in α_{1A} derived from rabbit. Generally, these subunits contain 4 internal homologous repeats (I-IV) each having six putative alpha helical membrane

spanning segments (S1-S6) with one segment (S4) having positively charged residues every 3rd or 4th amino acid. There are a minority of a splice variant exceptions. Between domains II and III there is a cytoplasmic domain which is believed to mediate excitation-contraction coupling in α_{1S} and which ranges from 100-400 amino acid residues among the subtypes. The domains I-IV make up roughly 2/3 of the molecule and the carboxy terminus adjacent to the S6 region of domain IV is believed to be on the intracellular side of the calcium channel. There is a consensus motif (QQ-E-L-GY-WI-E) in all of the subunits cloned and described in Stea, A., *et al.* (supra) downstream from the domain I S6 transmembrane segment that is a binding site for the β subunit.

PCT publication WO 98/38301, which describes the work of the inventors herein, and which is incorporated herein by reference, reports the first description of the molecular composition of T-type calcium channel α_1 subunits. The present application describes full-length genes for 3 mammalian subtypes, α_{1G} , α_{1H} , and α_{1I} associated with T-type calcium channels.

In some expression systems the high threshold α_1 subunits alone can form functional calcium channels although their electrophysiological and pharmacological properties can be differentially modulated by coexpression with any of the four β subunits. Until recently, the reported modulatory affects of β subunit coexpression were to mainly alter kinetic and voltage- dependent properties. More recently it has been shown that β subunits also play crucial roles in modulating channel activity by protein kinase A, protein kinase C and direct G-protein interaction. (Bourinet, *et al.* (1994) "Voltage-dependent facilitation of a neuronal α_1C L-type calcium channel." *EMBO J.* 13: 5032-5039; Stea, *et al.* (1995) "Determinants of PKC- dependent modulation of a family of neuronal calcium channels." *Neuron* 15:929-940; Bourinet, *et al.* (1996) "Determinants of the G-protein-dependent opioid modulation of neuronal calcium channels." *Proc. Natl. Acad. Sci. (USA)* 93: 1486-1491.)

Because of the importance of calcium channels in cellular metabolism and human disease, it would be desirable to identify the remaining classes of α_1 subunits, and to develop expression systems for these subunits which would permit the study and characterization of these calcium channels, including the study of pharmacological modulators of calcium channel function.

DISCLOSURE OF THE INVENTION

The present invention provides sequences for a novel mammalian calcium channel subunits of T-type calcium channels, which we have labeled as α_{1G} , α_{1H} and α_{1I} subunits. Knowledge of the sequences of these calcium channel subunits may be used in the development of probes for mapping the distribution and expression of the subunits in target tissues. In addition, as the molecular structure of the α_1 subunits of these T-type calcium channels has been elucidated, it is possible to identify those portions which reside extracellularly and thus to design peptides to elicit antibodies which can be employed to assess the location and level of expression of T-type calcium channels. In addition, these subunits, either alone or assembled with other proteins, can produce functional calcium channels, which can be evaluated in model cell lines to determine the properties of the channels containing the subunits of the invention. These cell lines can be used to evaluate the effects of pharmaceuticals and/or toxic substances on calcium channels incorporating α_{1G} , α_{1H} and α_{1I} subunits. The resulting identified compounds are useful in treating conditions where undesirable T-type calcium channel activity is present. These conditions include epilepsy, sleep disorders, mood disorders, cardiac hypertrophy and arrhythmia and hypertension, among others. In addition, antisense and triplex nucleotide sequences can be designed to inhibit the production of T-type calcium channels.

In a preferred embodiment the α_1 subunits are other than those encoded by SEQ. ID. NO: 17; in another preferred embodiment the α_1 subunits are other than those encoded by sequences that include SEQ. ID. NO: 19 and SEQ. ID. NO: 21. In another preferred embodiment, probes representing portions of or all of SEQ. ID. NOS. 1-22 or 13-21 are excluded.

BRIEF DESCRIPTION OF THE DRAWINGS

Figs. 1A and B show a comparison of the waveforms and current voltage relationship for α_{1G} ;

Figs. 2A and B show a comparison of the waveforms and current voltage relationship for α_{1I} calcium channels.

Fig. 3 shows a comparison of the steady state inactivation profiles of the α_{1G} and α_{1I} calcium channels.

Figs. 4A-C show a comparison of the inactivation kinetics of the α_{1G} and α_{1I} calcium channels.

Figures 5A and 5B show the construction of the human α_{1G} cDNA complete sequence from partial clones.

Figure 6 shows the nucleotide and deduced amino acid sequence of human T-type calcium channel α_{1G} .

Figure 7 shows a comparison of the waveforms and current voltage relationship for human α_{1G} calcium channel.

Figure 8 shows the characteristic pore pattern for T-type channels.

MODES OF CARRYING OUT THE INVENTION

The present invention includes the following aspects for which protection is sought:

(a) novel mammalian (including human) calcium channel subunits and DNA sequences encoding such subunits. Specifically, the invention encompasses an at least partially purified DNA molecule comprising a sequence of nucleotides that encodes an α_1 subunit of a T-type calcium channel, and such α subunits *per se*. It will be appreciated that polymorphic variations may be made or may exist in the DNA of some individuals leading to minor deviations in the DNA or amino acids sequences from those shown which do not lead to any substantial alteration in the function of the calcium channel. Such variations, including variations which lead to substitutions of amino acids having similar properties are considered to be within the scope of the present invention. Thus, in one embodiment, the present application claims DNA molecules which encode α_1 subunits of mammalian T-type calcium channels, and which hybridize under conditions of medium (or higher) hybridization stringency with one or another of the specific sequences disclosed in this application. This level of hybridization stringency is generally sufficient given the length of the sequences involved to permit recovery of the subunits within the scope of the invention from mammalian DNA libraries.

Alternatively, the T-type calcium channels of the invention are recognized by their functional characteristic of low voltage gating along with defined structural characteristics which classify them as α_1 calcium channel subunits and also characterize them as of the T-type. By virtue of the present invention, these characteristics have been elucidated as follows:

One distinguishing feature of the α_{1G} , α_{1H} and α_{1I} T-type channels over other types of calcium channels and sodium channels is that the pore region (P-region) in each of the four structural domains contains a diagnostic amino acid sequence implicated in channel permeability. Figure 8 shows that the T-type channels contain the residues glutamate/glutamate/aspartate/aspartate (single letter amino acid code: EEDD) in the P-regions of domains I-IV. In contrast, figure 8 shows that in sodium (Na) channels the P-region of the four domains contains the residues: aspartate/glutamate/lysine/alanine (single letter amino acid code: DEKA), while high threshold calcium channels such as the L-type channel contain the residues: glutamate/glutamate/glutamate/glutamate (single letter amino acid code: EEEE). The α_{1G} , α_{1H} and α_{1I} T-type channels are also distinct in this region compared to other types of ion channels including the *C. elegans* C11D2.6 and C27F2.3 and the rat NIC-channel (Figure 8).

A second distinguishing characteristic of the α_{1G} , α_{1H} and α_{1I} T-type channels compared to other types of calcium channels is that they do not contain a β subunit binding consensus sequence in the cytoplasmic linker separating domains I and II. In contrast, all high threshold calcium channels contain a consensus sequence (single letter amino acid code: QQ-E--L-GY--WI---E) shown to physically interact with the calcium channel β subunit (Pragnell, M., De Waard, M., Mori, Y., Tanabe, T., Snutch, T.P. & Campbell, K.P., 1994, Nature 368:67-70). Thus it appears the presence of a β subunit does not modify activity, nor is its presence required.

A third distinguishing characteristic of the (α_{1G} , α_{1H} and α_{1I} T-type channels is that they do not possess an EF-hand calcium binding motif in the region carboxyl to domain IV S6. In contrast, all high threshold calcium channels contain a consensus sequence that is closely related to the EF-hand domain found in certain calcium binding

proteins (de Leon, M., Wang, Y., Jones, L., Perez-Reyes, E., Wei, X., Soong, T.W., Snutch, T.P. & Yue, D.T., 1995, Science 270: 1502-1506).

Thus, as defined herein, "T-type calcium channel α_1 subunits" refers to subunits which contain these structural characteristics.

Alternatively, the T-type α_1 subunit molecules can be defined by homology to the human and rat nucleotide and amino acid sequences described herein. Thus, T-type α_1 subunits will typically have at least 50%, preferably 70% homology in terms of amino acid sequence or encoding nucleotide sequence to the sequences set forth in SEQ ID NOS. 23-28 herein or those shown in Figure 6. Preferably, the homology will be at least 80%, more preferably 90%, and most preferably 95%, 97%, 98% or 99%.

Relative homology may also be defined in terms of specific regions; as set forth above, certain regions of T-type channel α_1 subunits have very high homologies while other regions, such as the cytoplasmic region between domains II and III have less homology. Thus, T-type α_1 subunits will have over 75% homology; preferably over 85% or over 95% homology, more preferably over 98% homology in domains I-IV to those of SEQ. ID. NOS. 23-28 or Figure 6. The degree of homology in the cytoplasmic region between domains II and III may be substantially less, *e.g.*, only 25% homology, preferably, 50% homology or more preferably 60% homology. Similarly, the intracellular region downstream of domain IV may be less homologous than within domains I-IV.

(b) polynucleotide sequences useful as probes in screening human cDNA libraries for genes encoding these novel calcium channel subunits. These probes can also be used in histological assay to determine the tissue distribution of the novel calcium channel subunits.

As set forth above, the elucidation herein of the structural features of T-type subunits permits the selection of appropriate probes by selecting portions of the encoding nucleotide sequence that are particularly characteristic of this type. As set forth above, for example, T-type subunits have particular patterns of amino acids in the pore forming units as set forth in Figure 8. Alternatively, multiple probes might be used to distinguish other subunits, such as probes which represent the β -binding domain missing from the T-

type α_1 subunits combined with a probe representing a consensus sequence for calcium channel α subunits in general.

(c) at least partially purified α_1 subunits and related peptides for mammalian T-type calcium channels. These proteins and peptides can be used to generate polyclonal or monoclonal antibodies to determine the cellular and subcellular distribution of T-type calcium channel subunits.

Again, by virtue of the elucidation of the amino acid sequence of T-type α_1 subunits, it is well within the ordinary skill in the art to determine which regions of the channel are displayed extracellularly and to select these regions for the generation of antibodies.

(d) eukaryotic cell lines expressing the novel calcium channel subunits. These cell lines can be used to evaluate compounds as pharmacological modifiers of the function of the novel calcium channel subunits.

(e) a method for evaluating compounds as pharmacological modifiers of the function of the novel calcium channel subunits using the cell lines expressing those subunits alone or in combination with other calcium channel subunits.

(f) Use of the compounds identified as set forth above for the treatment of conditions which are associated with undesired calcium channel activity.

These diseases include, but are not limited to; epilepsy, migraine, ataxia, schizophrenia, hypertension, arrhythmia, angina, depression, and Parkinson's disease; characterization of such associations and ultimately diagnosis of associated diseases can be carried out with probes which bind to the wild-type or defective forms of the novel calcium channels.

T-type channels in particular are responsible for rebound burst firing in central neurons and are implicated in normal brain functions such as slow-wave sleep and in neurological disorders such as epilepsy and mood disorders. They are also important in pacemaker activity in the heart, hormone secretion and fertilization, and are associated with disease states such as cardiac hypertrophy and hypertension.

As used in the specification and claims of this application, the term "T-type calcium channel" refers to a voltage-gated calcium channel having a low activation voltage, generally less than -50 mV, and most commonly less than -60 mV. T-type

calcium channels also exhibit comparatively negative steady-state inactivation properties and slow deactivation kinetics. The terms " α_1 subunit" or " α_1 calcium channel" refer to a protein subunit of a calcium channel which is responsible for pore formation and contains the voltage sensor and binding sites for calcium channel agonists and antagonists. Such subunits may be independently functional as calcium channels or may require the presence of other subunit types for complete functionality.

As used in the specification and claims of this application, the phrase "at least partially purified" refers to DNA or protein preparations in the which the specified molecule has been separated from adjacent cellular components and molecules with which it occurs in the natural state, either by virtue of performing a physical separation process or by virtue of making the DNA or protein molecule in a non-natural environment in the first place. The term encompasses cDNA molecules and expression vectors.

In accordance with the present invention, we have identified mammalian DNA sequences which code for novel T-type calcium channel α_1 subunits. These subunits are believed to represent new types of α_1 subunits of mammalian voltage-dependent calcium channels which have been designated as types α_{1G} , α_{1H} and α_{1I} .

A Bacterial Artificial Chromosome (BAC) sequence (bK206c7) was identified from sequences in Sanger Genome Sequencing Center (Cambridge, U.K.) and the Washington University Genome Sequencing Center (St. Louis, MO) that contains a nucleotide sequence encoding the α_{1I} subunit of human T-type calcium channel. The rationale for this identification is set forth in WO 98/38301, incorporated herein by reference. The relevant nucleotide sequence and the translated amino acid sequence containing 1854 amino acids are set forth in SEQ ID NOS:17 and 18.

As described in WO 98/38031, using PCR cloning techniques to identify relevant sequences within a human brain total RNA preparation, we confirmed that the novel α_{1I} calcium channel subunit is present in human brain. Subcloning of the 567 nt PCR product (SEQ. ID NO. 19, amino acids SEQ. ID NO. 20) and subsequent sequencing thereof showed that this product corresponds to the derived sequence from the bK206c7 BAC genomic sequence, the nucleotide sequence of which is given as SEQ ID NO. 17 (amino acid sequence SEQ. ID NO.18). The same experiment was performed using a rat

brain RNA preparation and resulted in recovery of a substantially identical PCR product. (SEQ ID. NO. 21). The protein encoded by the rat PCR product (SEQ ID NO. 22) is 96% identical to the human PCR product (SEQ. ID NO. 20).

These sequences, which encode a partial subunit were used as a basis for constructing full length human or rat α_{11} clones. Briefly, the subcloned α_{11} PCR product is radiolabeled by random hexamer priming according to standard methods (See, Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning, A Laboratory Manual*. Cold Spring Harbor Press) and used to screen commercial human brain cDNA libraries (Stratagene, La Jolla, CA). The screening of cDNA libraries follows standard methods and includes such protocols as infecting bacteria with recombinant lambda phage, immobilizing lambda DNA to nitrocellulose filters and screening under medium hybridization stringency conditions with radiolabeled probe. cDNA clones homologous to the probe are identified by autoradiography. Positive clones are purified by sequential rounds of screening.

Following this protocol, most purified cDNA's are likely to be partial sequence clones due to the nature of the cDNA library synthesis. Full length clones are constructed from cDNA's which overlap in DNA sequence. Restriction enzyme sites which overlap between cDNAs are used to ligate the individual cDNA's to generate a full-length cDNA. For subsequent heterologous expression, the full-length cDNA is subcloned directly into an appropriate vertebrate expression vector, such as pcDNA-3 (Invitrogen, San Diego, CA) in which expression of the cDNA is under the control of a promoter such as the CMV major intermediate early promoter/enhancer. Other suitable expression vectors include, for example, pMT2, pRC/CMV, pcDNA3.1 and pCEP4.

Following these protocols, full length mammalian α_{1G} , α_{1H} and α_{1I} calcium channel subunit cDNAs were isolated by using the 567 base pair human fragment (SEQ. ID NO. 19) to screen a rat brain cDNA library. Sequencing of the recovered sequences identified the three distinct classes of calcium channel subunits which have been denominated herein as α_{1G} , α_{1H} and α_{1I} subunits. For each class of subunit, complete sequencing of the largest cDNA confirmed that it represented only a portion of the predicted calcium channel coding region. Complete sequences for the three new subunits were obtained by rescreening the rat brain cDNA library with probes derived from the

partial length cDNAs to obtain overlapping segments. These segments were combined to form a complete gene by restriction digestion and ligation. The complete cDNA sequences of the rat α_{1G} , α_{1H} and α_{1I} subunits are given by SEQ. ID NOS. 23, 25 and 27, respectively. Corresponding amino acid sequences are given by SEQ. ID NOS. 24, 26 and 28. The same techniques are employed to recover human sequences by screening of a human or other mammalian library. Thus, for example, partial length human sequences for α_{1G} and α_{1H} T-type calcium channels have been recovered using the same probe (SEQ. ID NO. 19) and the full length rat α_{1I} cDNA (SEQ. ID. NO. 27) has been used to recover a partial length DNA encoding a human α_{1I} T-type calcium channel. The DNA and amino acid sequences for these partial length human calcium channels are given by SEQ. ID NOS. 30-35. A complete coding sequence for human α_{1G} was also obtained and is set forth, along with the deduced amino acid reference, in Figure 6.

Once the full length cDNA is cloned into an expression vector, the vector is then transfected into a host cell for expression. Suitable host cells include *Xenopus* oocytes or mammalian cells such as human embryonic kidney cells as described in International Patent Publication No. WO 96/39512 which is incorporated herein by reference and Ltk cells as described in US Patent No. 5,386,025 which is incorporated herein by reference. Transfection into host cells may be accomplished by microinjection, lipofection, glycerol shock, electroporation calcium phosphate or particle-mediated gene transfer. The vector may also be transfected into host cells to provide coexpression of the novel α_1 subunits with other α subunits, such as an $\alpha_{2\delta}$ subunit or γ subunit.

To confirm that the three full length cDNAs (SEQ. ID NOS. 23, 25 and 27) encoded functional calcium channels, the α_{1G} and α_{1I} cDNAs were transiently transfected into human embryonic kidney cells and evaluated using electrophysiological recording techniques. The results are consistent with a role of these subunits in native T-type channels in nerve, muscle and endocrine cells. Similarly, a full length clone encoding human α_{1G} T-type subunit was recovered and verified to have the characteristic properties of T-type channels.

The resulting cell lines expressing functional calcium channels including the novel α_1 subunits of the invention can be used test compounds for pharmacological activity with respect to these calcium channels. Thus, the cell lines are useful for

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and 27 or Figure 6 or distinctive portions thereof which do not exhibit non-discriminatory levels of homology with other types of calcium channel subunits may also be used for mapping the distribution of α_{1G} , α_{1H} and α_{1I} calcium channel subunits within a tissue sample. This method follows normal histological procedures using a nucleic acid probe, and generally involves the steps of exposing the tissue to a reagent comprising a directly or indirectly detectable label coupled to a selected DNA fragment, and detecting reagent that has bound to the tissue. Suitable labels include fluorescent labels, enzyme labels, chromophores and radio-labels.

Heterologous Expression of Mammalian T-type Calcium Channels in Cells

A. Transient Transfection in Mammalian Cells

Host cells, such as human embryonic kidney cells, HEK 293 (ATCC# CRL 1573) are grown in standard DMEM medium supplemented with 2 mM glutamine and 10% fetal bovine serum. HEK 293 cells are transfected by a standard calcium-phosphate-DNA co-precipitation method using a full-length mammalian α_1 T-type calcium channel cDNA (for example, SEQ. ID. NO. 27) in a vertebrate expression vector (for example see Current protocols in Molecular Biology). The α_{1I} calcium channel cDNA may be transfected alone or in combination with other cloned subunits for mammalian calcium channels, such as $\alpha_{2\delta}$ and β or γ subunits, and also with clones for marker proteins such the jellyfish green fluorescent protein.

Electrophysiological Recording: After an incubation period of from 24 to 72 hrs the culture medium is removed and replaced with external recording solution (see below). Whole cell patch clamp experiments are performed using an Axopatch 200B amplifier (Axon Instruments, Burlingame, CA) linked to an IBM compatible personal computer equipped with pCLAMP software. Microelectrodes are filled with 3 M CsCl and have typical resistances from 0.5 to 2.5 Mohms. The external recording solution is 2 mM BaCl_2 , 1 mM MgCl_2 , 10 mM HEPES, 40 mM TEACl, 10 mM Glucose, 92 mM CsCl, (pH 7.2). The internal pipette solution is 105 mM CsCl, 25 mM TEACl, 1 mM CaCl_2 , 11 mM EGTA, 10 mM HEPES (pH 7.2). Currents are typically elicited from a holding potential of -100 mV to various test potentials. Data are filtered at 1 kHz and recorded directly on the hard-drive of a personal computer. Leak subtraction is carried out on-line

using a standard P/5 protocol. Currents are analyzed using pCLAMP versions 5.5 and 6.0. Macroscopic current-voltage relations are fitted with the equation $I = \frac{1}{1 + \exp(-(V_m - V_h)/S)} \times G - (V_m - E_{rev})$, where V_m is the test potential, V_h is the voltage at which half of the channels are activated, and S reflects the steepness of the activation curve and is an indication of the effective gating charge movement. Inactivation curves are normalized to 1 and fitted with $I = (1 / (1 + \exp((V_m - V_h)/S)))$ with V_m being the holding potential. Single channel recordings are performed in the cell-attached mode with the following pipette solution (in mM): 100 BaCl₂, 10 HEPES, pH 7.4 and bath solution: 100 KCl, 10 EGTA, 2 MgCl₂, 10 HEPES, pH 7.4.

B. Transient Transfection in *Xenopus* Oocytes

Stage V and VI *Xenopus* oocytes are prepared as described by Dascal, *et al* (1986), Expression and modulation of voltage-gated calcium channels after RNA injection into *Xenopus* oocytes. Science 231:1147-1150. After enzymatic dissociation with collagenase, oocytes nuclei are microinjected with the human α_{1I} calcium channel cDNA expression vector construct (approximately 10 ng DNA per nucleus) using a Drummond nanoject apparatus. The α_{1I} calcium channel may be injected alone, or in combination with other mammalian calcium channel subunit cDNAs, such as the $\alpha_2\delta$ and β_1b and γ subunits. After incubation from 48 to 96 hrs macroscopic currents are recorded using a standard two microelectrode voltage-clamp (Axoclamp 2A, Axon Instruments, Burlingame, CA) in a bathing medium containing (in mM): 40 Ba(OH)₂, 25 TEA-OH, 25 NaOH, 2 CsOH, 5 HEPES (pH titrated to 7.3 with methane-sulfonic acid). Pipettes of typical resistance ranging from 0.5 to 1.5 Mohms are filled with 2.8M CsCl, 0.2M CsOH, 10mM HEPES, 10mM BAPTA free acid. Endogenous Ca (and Ba) - activated Cl currents are suppressed by systematically injecting 10-30 nl of a solution containing 100mM BAPTA-free acid, 10mM HEPES (pH titrated to 7.2 with CsOH) using a third pipette connected to a pneumatic injector. Leak currents and capacitive transients are subtracted using a standard P/5 procedure.

Construction of Stable Cell Lines Expressing Mammalian T-type Calcium Channels

Mammalian cell lines stably expressing human α_{11} calcium channels are constructed by transfecting the α_{11} calcium channel cDNA into mammalian cells such as HEK 293 and selecting for antibiotic resistance encoded for by an expression vector.

5 Briefly, a full-length mammalian T-type calcium channel α_1 subunit cDNA (for example SEQ. ID NO. 27) subcloned into a vertebrate expression vector with a selectable marker, such as the pcDNA3 (InvitroGen, San Diego, CA), is transfected into HEK 293 cells by calcium phosphate coprecipitation or lipofection or electroporation or other method according to well known procedures (Methods in Enzymology, Volume 185, Gene
10 Expression Technology (1990) Edited by Goeddel, D.V.). The α_{11} calcium channel may be transfected alone, or in combination with other mammalian calcium channel subunit cDNAs, such as the α_2 - δ and β_{1b} subunits, either in a similar expression vector or other type of vector using different selectable markers. After incubation for 2 days in nonselective conditions, the medium is supplemented with Geneticin (G418) at a
15 concentration of between 600 to 800 ug/ml. After 3 to 4 weeks in this medium, cells which are resistant to G418 are visible and can be cloned as isolated colonies using standard cloning rings. After growing up each isolated colony to confluency to establish cell lines, the expression of α_{11} calcium channels can be determined at with standard gene expression methods such as Northern blotting, RNase protection and reverse-transcriptase
20 PCR.

The functional detection of α_{11} calcium channels in stably transfected cells can be examined electrophysiologically, such as by whole patch clamp or single channel analysis (see above). Other means of detecting functional calcium channels include the use of radiolabeled ^{45}Ca uptake, fluorescence spectroscopy using calcium sensitive dyes
25 such as FURA-2, and the binding or displacement of radiolabeled ligands that interact with the calcium channel.

Example 1

Partial Rat and Human Subunits

In order to recover mammalian sequences for novel calcium channels, the 567 base pair partial length human brain α_{1I} cDNA described in WO 98/3801 was gel-purified, radio-labeled with ^{32}P dATP and dCTP by random priming (Feinberg, *et al.*, 1983, *Anal. Biochem.* 132: 6-13) and used to screen a rat brain cDNA library constructed in the phase vector Lambda Zapp II. (Snutch *et al.*, 1990, *Proc Natl Acad Sci (USA)* 87: 3391-3395). Screening was carried out at 62°C in 5XSSPE (1XSSPE= 0.18 M NaCl; 1mM EDTA; 10 mM sodium phosphate, pH=7.4 0.3% SDS, 0.2 mg/ml denatured salmon sperm DNA). Filters were washed at 62°C in 0.2X SSPE/0.1% SDS. After three rounds of screening and plaque purification, positive phages were transformed into Bluescript phagemids (Stratagene, La Jolla, CA) by *in vivo* excision.

Double stranded DNA sequencing on the recombinant phagemids was performed using a modified dideoxynucleotide protocol (Biggin *et al.*, 1983, *Proc Natl Acad Sci (USA)* 80:3963-3965) and Sequenase version 2.1 (United States Biochemical Corp.). DNA sequencing identified three distinct classes of calcium channel α_1 subunits: designated as α_{1G} , α_{1H} and α_{1I} calcium channel subunits.

For each class of calcium channel α_1 subunit, the largest cDNA was completely sequenced and determined to represent only a portion of the predicted calcium channel coding region. In order to isolate the remaining portions of α_{1G} and α_{1I} calcium channel subunits, the α_{1G} clone was digested with HindIII and SpeI. The resulting 540 base pair fragment was gel purified, radiolabeled with ^{32}P dATP and dCTP by random priming and used to rescreen the rat brain cDNA library as described above. The sequence of the 540 base pair α_{1G} screening probe used is given by SEQ. ID NO. 29. Other sequences spanning regions of distinctiveness within the sequences for the subunits could also be employed.

Double-stranded DNA sequencing of the purified recombinant phagemids showed that additional α_{1G} , α_{1H} and α_{1I} calcium channel subunit cDNAs overlapped with the original partial length cDNAs and together encoded complete protein coding regions as well as portions of their respective 5' and 3' non-coding untranslated regions.

To recover further human sequences for the novel α_{1G} and α_{1H} calcium channels, the 567 base pair partial length human brain α_{1I} cDNA (SEQ. ID. NO: 19) was radio-labeled with ^{32}P dATP and dCTP by random priming and used to screen a commercial human thalamus cDNA library (Clontech). Hybridization was performed overnight at 65°C in 6 X SSPE; 0.3% SDS; 5X Denhardt's. Filters were washed at 65°C in 0.1 X SSPE/ 0.3% SDS. After four rounds of screening and plaque purification, positive phages were selected, DNA prepared and the insert cDNA excised from the lambda vector by digestion with Eco R1 restriction enzyme. The excised cDNA was subcloned into the plasmid Bluescript KS (Stratagene, La Jolla, CA) and the DNA sequence determined using a modified dideoxynucleotide protocol and Sequence version 2.1. The partial length α_{1G} cDNA isolated consisted of 2212 base pairs of which 279 base pairs were 5' noncoding and 1,933 base pairs were coding region representing 644 amino acids (SEQ. ID NOS. 30, 31). The partial α_{1H} cDNA isolated consisted of 1,608 base pairs of which 53 base pairs were 5' noncoding and 1,555 were coding region representing 518 amino acids (SEQ. ID NOS. 32, 33).

To recover further human sequences for the novel α_{1I} calcium channel, the full-length rat brain α_{1I} cDNA (SEQ. ID. NO: 27) (See Example 2) was radio-labeled ^{32}P dATP and dCTP by random priming and used to screen a commercial human hippocampus cDNA library (Stratagene). Hybridization was performed overnight at 65 °C in 6 X SSPE; 0.3% SDS; 5X Denhardt's. Filters were washed at 65 °C in 0.1 X SSPE/ 0.3% SDS. After four rounds of screening and plaque purification, positive phages were transformed into Bluescript phagemids (Stratagene, LA Jolla, CA) by *in vitro* excision. The excised cDNA DNA sequence was determined using a modified dideoxynucleotide protocol and Sequenase version 2.1. The partial α_{1I} cDNA isolated consisted of 1,080 base pairs of coding region representing 360 amino acids (SEQ. ID NOS. 34, 35).

Example 2

Full Length Rat Subunits

Double-stranded DNA sequencing of the purified recombinant phagemids from rat brain showed that additional α_{1G} and α_{1I} calcium channel cDNAs overlapped with the original partial length cDNAs and together encoded complete protein coding regions as

well as portions of their respective 5' and 3' non-coding untranslated regions. (SEQ. ID NOS. 23 and 27, respectively) In addition to the α_{1G} and α_{1I} calcium channel classes, DNA sequencing of the recombinant phagemids also identified a third class of calcium channel α_1 subunit: designated as the α_{1H} calcium channel subunit. The partial length α_{1H} calcium channel cDNAs overlapped and together encoded a complete α_{1H} coding region as well as portions of the 5' and 3' untranslated regions (SEQ. ID. NO. 25).

Electrophysiological studies were performed on transiently-transfected human embryonic kidney cells (HEK-tsa201) prepared using the general protocol above. Transfection was carried out by standard calcium phosphate precipitation. (Okayama *et al.*, 1991, *Methods in Molec. Biol.*, Vol. 7, ed. Murray, E.J.). For maintenance, HEK-tsa201 cells were cultured until approximately 70% confluent, the media removed and cells dispersed with trypsin and gentle trituration. Cells were then diluted 1:10 in culture medium (10% FBS, DMEM plus L-glutamine, pen-stp) warmed to 37°C and plated onto tissue culture dishes. For transient transfection, 0.5 mM CaCl_2 was mixed with a total of 20 μg of DNA (consisting of 3 μg of either rat brain α_{1G} or α_{1I} calcium channel cDNA, 2 μg of CD8 plasmid marker, and 15 μg of Bluescript plasmid carrier DNA). The DNA mixture was mixed thoroughly and then slowly added dropwise to 0.5 ml of 2 times HeBS (274 mM NaCl, 20mM D-glucose, 10mM KCl, 1.4 mM Na_2HPO_4 , 40 mM Hepes (pH=7.05). After incubation at room temperature for 20 min, 100 μl of the DNA mixture was slowly added to each dish of HEK-tsa201 cells and then incubated at 37°C for 24 to 48 hours in a tissue culture incubator (5% CO_2).

Positive transfectant cells were identified visually by addition of 1 μl of mouse CD8 (Lyt2) Dynabeads directly to the recording solution and gentle swirling to mix. Whole cell patch clamp readings were carried out with an Axopatch 200A amplifier (Axon Instruments) as described previously. (Zamponi *et al.*, 1997, *Nature* 385: 442-446). The external recording solution was 2 mM CaCl_2 , 1 mM MgCl_2 , 10 mM HEPES, 40 mM TEA-Cl, 10 mM glucose, 92 mM CsCl, pH=7.2 with TEA-hydroxide. The internal pipette solutions was 105 mM CsCl, 25 mM TEA-Cl, 1mM CaCl_2 , 11 mM EGTA, 10 mM HEPES, pH 7.2 with NaOH.

For determination of current-voltage (I-V) relationships, cells were held at a resting potential of -100 mV and then stepped to various depolarizing test potentials. For

steady-state inactivation, cells were held at various potentials for 20 sec. and currents recorded during a subsequent test pulse to the peak potential of the I-V. Leak currents and capacitative transients were subtracted using a P/5 procedure.

Figs. 1-4 show the results obtained for HEK cells transfected with and expressing the cDNA of sequences ID Nos. 23 and 27, which correspond to the subunits designated as α_{1G} and α_{1I} . Figs. 1A and B and 2A and B shows a comparison of the waveforms and current-voltage relationship for the two channel subunit types. In the presence of recording solution containing 2mM Ca^{2+} , both the α_{1G} and α_{1I} channel subunits exhibit activation properties consistent with native T-type calcium currents. Figs. 1A and 2A show the subunit calcium current from a cell held at -120 mV and depolarized to a series of test potentials. Figs. 1B and 2B show the magnitude of the calcium current. From a holding potential of -110 mV, both channel first activate at approximately -70 mV and peak currents are obtained between -40 and -50 mV. Upon depolarization to various test potentials, the current waveforms of the α_{1G} and α_{1I} calcium channels exhibit an overlapping pattern characteristic of native T-type channels in nerve, muscle and endocrine cells.

Fig. 3 shows steady-state inactivation profiles for the α_{1G} and α_{1I} calcium channels in HEK 293 cells transiently transformed with full length cDNAs (SEQ ID NOS. 23 or 27) for α_{1G} or α_{1I} subunits. Steady state inactivation properties were determined by stepping from -120 mV to prepulse holding potentials between -120 mV and -50 mV for 15 sec., prior to a test potential of -30 mV. The data are plotted as normalized whole cell current versus prepulse holding potential and show that α_{1G} exhibits a V_{50} of approximately -85 mV and α_{1I} a V_{50} of approximately -93 mV. Thus, consistent with native T-type calcium channels, both of the α_{1G} and α_{1I} calcium channels exhibit pronounced steady-state inactivation at negative potentials.

Figs. 4A-C show a comparison of the voltage-dependent deactivation profiles of the α_{1G} and α_{1I} calcium channels. HEK 293 cells were transiently transfected with either an α_{1G} or α_{1I} subunit cDNA (SEQ. ID NO. 23 or 27). The deactivation properties of α_{1G} were determined by stepping from a holding potential of -100 mV to -40mV for 9 msec, and then to potentials between -120 mV and -45 mV. The deactivation properties of α_{1I} were determined by stepping from a holding potential of -100 mV to -40 mV for 20

msec, and then to potentials between -120 mV and -45 mV. Both channels exhibit slow deactivation kinetics compared to typical high-threshold channels, and is consistent with the α_{1G} and α_{1I} subunits being subunits for T-type calcium channels

Example 3

Cloning of a Full Length cDNA for the Human α_{1G} T-Type Calcium Channel Subunit

Materials and Methods:

A full length cDNA encoding the human α_{1G} subunit was constructed from 5 overlapping clones (Figure 1B) isolated from a human thalamus cDNA library constructed in λ gt11 vector (Clontech, Cat#HL5009b).s

Three λ gt11 cDNA clones were isolated by conventional filter hybridization.

Clone 1 was identified by hybridization to a 567 bp cDNA probe (SEQ. ID. NO: 19) containing the transmembrane region S4 to S6 of domain I of the previously cloned human neuronal α_{1I} T-type calcium channel subunit. Clones HG10-1112 and HG5-1211 were identified by hybridization to a 1265 bp cDNA probe of the rat α_{1H} T-type calcium channel subunit spanning domain II and part of the II-III intracellular loop. cDNA probes were 32 P-dCTP labeled by random priming using a Multiprime DNA labeling system (Amersham Pharmacia). Plaque lifts using H-bond nylon membranes were done in duplicate following the standard protocols supplied by manufacturer (Amersham Pharmacia). Hybridization was performed for at least 16 hrs at 65°C for clone 1 and for at least 16 hrs at 58°C, clones HG10-1112 and HG5-1211. Membranes were washed in 0.1X SSC/0.3% SDS at 62°C for clone 1 and 0.2X SSC/0.1% SDS at 58°C clones HG10-1112 and HG5-1211. Blots were exposed to BioMax MS Kodak film with Kodak HE intensifying screens for at least 48 hrs at -80°C. Double positive plaques were isolated and re-screened to isolate single clones according to the procedure above.

Bacteriophage DNA's were then isolated according to the λ gt11 library User Manual (Clontech). Clone 1 cDNA insert was excised with EcoRI (NEB) and subcloned into pBluescriptKS (Stratagene). Clones HG10-1112 and HG5-1211 cDNA inserts were excised from λ DNA with Not I (NEB) and subcloned into the Not I site of pBluescriptKS. Plasmids with cDNA inserts were transformed by electroporation into

XL-I *E. coli* host strain bacteria and sequenced using universal reverse and forward primers according to Sanger double stranded DNA sequencing method in combination with automatic sequencing ABI 100 PRISM model 377 Version 3.3 (PE Biosystems).

Clone 1 was identified as a human α_{1G} subunit containing the 5'UTR and 1933 bp of the in-frame coding region, including part of the intracellular I-II loop. Clone HG10-1112 was identified as a human α_{1G} subunit of 3915 bp, spanning DomainI (S5-S6) to the III-IV loop. Clone HG5-1211 was identified as human α_{1G} subunit of 3984 bp containing the I-II linker and C-terminus.

For expression in HEK cells, removal of 5' UTR from clone 1 was achieved by replacing 5'UTR DNA fragment flanked by Hind III/SacII restriction sites with 5'end - 291 bp cDNA fragment, containing translation start site and an incorporated Hind III site for subsequent cloning into pcDNA3.1 (Invitrogen). Following PCR conditions were used: 94°C -30 sec, 45°C -30 sec, 72°C -30 sec for 5 cycles and followed by 94°C -30 sec, 48°C -30 sec, 72°C -30 sec for 20 cycles (Bio-rad Gene Cyclor). The cDNA fragment was subcloned into p-Gem-T-Easy plasmid vector (Promega) and the DNA sequence determined.

The remaining region of the 3' α_{1G} subunit cDNA was obtained using the PCR method on a human thalamus cDNA library with primers MD19-sense (5'GCG TGG AGC TCT TTG GAG 3') and G26- antisense (5' GCA CCC AGT GGA GAA AGG TG 3'). The PCR protocol used was 94°C -30 sec, 58°C -30 sec, 72°C -30 sec for 25 cycles (Bio-rad Gene Cyclor). A cDNA fragment of 1617 bp was subcloned into p-Gem-T-Easy plasmid vector (Promega) and sequenced. The 3'PCR cDNA was identified as a human α_{1G} subunit spanning from Domain IV-S5 to the carboxyl terminus including the stop codon.

Unique restriction sites (Figures 5A and B) of the partial cDNA clones were used to construct the full length human α_{1G} T-type calcium channel in pcDNA3.1 Zeo (+) (Invitrogen) mammalian expression vector.

The complete nucleotide and amino acid sequences are shown in Figure 6.

In order to determine the functional properties of the human α_{1G} channel standard calcium-phosphate transfection was used to transiently express the channel in HEK ts201

cells. Cells were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum, 200 U/ml penicillin and 0.2 mg/ml streptomycin at 37°C with 5% CO₂. At 85% confluency cells were split with 0.25% trypsin/1 mM EDTA and plated at 10% confluency on glass coverslips. At 12 hours the medium was replaced and the cells transiently transfected using a standard calcium phosphate protocol and the α_{1G} calcium channel cDNA. Fresh DMEM was supplied and the cells transferred to 28°C/5% CO₂. Cells were incubated for 1 to 2 days prior to whole cell recording. Whole cell patch recordings were performed using an Axopatch 200B amplifier (Axon Instruments) linked to an IBM compatible personal computer equipped with pCLAMP version 7.0 software. The intrapipette solution contained (in mM): 105 CsCl, 25 CsCl, 1 CaCl₂, 11 EGTA, 10 HEPES, pH 7.2. The extracellular solution contained (in mM): 40 TEA-Cl, 2 CaCl₂, 1 MgCl₂, 92 CsCl, 10 glucose, 10 HEPES, pH 7.2.

Figure 7 shows that the human α_{1G} cDNA encodes a calcium channel with typical properties of a T-type current. The left panel illustrates representative current traces obtained from a holding potential of -100 mV to test pulses potentials of -90 mV to +20 mV. The traces show a typical crossover pattern and considerable inactivation during the test pulse, both of which are consistent with native T-type channels. The right panel shows a plot of the peak whole current at various test potentials and indicates that the human α_{1G} cDNA first activates near -60 mV with maximal current near -40 mV, which is also consistent with native low-threshold T-type calcium channels.